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SHORT NOTE

Cryopreservation of in vitro-produced *Rhizophagus* species has minor effects on their morphology, physiology, and genetic stability

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Abstract Cryogenic storage is considered to be the most convenient method to maintain phenotypic and genetic stability of organisms. A cryopreservation technique based on encapsulation-drying of in vitro-produced arbuscular mycorrhizal fungi has been developed at the Glomeromycota In Vitro Collection. In this study, we investigated fungal morphology (i.e., number and size of spores, number of branched absorbing structures (BAS), hyphal length, and number of anastomosis per hyphal length), activity of acid phosphatase and alkaline phosphatase in extraradical hyphae, and variation in amplified fragment length polymorphism (AFLP) profiles of in vitro-produced isolates of five *Rhizophagus* species maintained by cryopreservation for 6 months at −130 °C and compared to the same isolates preserved at 27 °C. Isolates were stable after 6 months cryopreservation. Comparing isolates, the number of BAS increased significantly in one isolate, and hyphal length decreased significantly in another isolate. No other morphological variable was impacted by the mode of preservation. Phosphatase activities in extraradical hyphae and AFLP profiles were not influenced by cryopreservation. These findings indicate that cryopreservation at −130 °C of encapsulated-dried and in vitro-produced *Rhizophagus* isolates (i.e., *Rhizophagus irregularis*, *Rhizophagus fasciculatus*, *Rhizophagus diaphanous*, and two undefined isolates) is a suitable alternative for their long-term preservation.

Keywords Arbuscular mycorrhizal fungi · Cryopreservation · Encapsulation-drying · Genetic stability · Phosphatase activity · Amplified fragment length polymorphism (AFLP)

Introduction

Arbuscular mycorrhizal fungi (AMF) play an important role in plant biodiversity, variability of ecosystems, agricultural productivity, and plant protection against biotic and abiotic stresses (Van der Heijden and Scheublin 2007). They are used in many national and international programs to improve crop yield and reduce the impact of environmental a/biotic stresses, while limiting the use of chemical inputs (Azcon-Aguilar and Barea 1996). Preservation of their genetic, phenotypic, and physiological stability over long periods is thus required. Currently, AMF are maintained in collection in pot cultures and in vitro on root organs using serial sub-cultivation. These methods are time consuming and labor intensive. The risk of contamination is not negligible for the pot-cultured isolates, while the phenotypic stability and infectivity of the isolates cannot be formally guaranteed over years of routine maintenance for the in vitro material (Plenchette et al. 1996).

An alternative method to the in vitro serial sub-cultivation is cryopreservation at ultra-low temperature. Cryopreservation is considered as the most reliable long-term storage method for most filamentous fungi (Smith 1998). It requires limited space for storage and obviates the need for labor-intensive sub-cultivation. At ultra-low temperature, cell division and metabolic activities are arrested. The biological material may

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thus be preserved for an indefinite period of time. Recently, Lalaymia et al. (2012) succeeded in the cryopreservation of 12 AMF isolates belonging to the genus *Rhizophagus* using encapsulation-drying for several months at −130 °C. This method represents therefore a major breakthrough for culture collections. However, before large-scale utilization, the impact of cryopreservation on morphological, physiological, and genetic stability of AMF isolates should be assessed. A number of studies have reported modifications in fungal characteristics after cryopreservation. For instance, Smith (1998) observed by cryomicroscopy, after cooling at −50 °C, plasmolysis, and a 70 % diameter reduction of hyphae of *Thanatephorus* species which are ectomycorrhizal (ECM) fungi. *Scleroderma flavidum*, another ECM fungus, survived cryopreservation but had weak regrowth capacity (Corbery and LeTacon 1997). Obase et al. (2011) observed a reduced hyphal growth or complete inhibition of several ECM fungi after 6 months storage at −70 °C. Finally, Ryan et al. (2001) detected genetic polymorphism in two isolates of *Metarhizium anisopliae* after storage at −196 °C. From these examples, it is not excluded that similar problems may arise with AMF isolates following cryopreservation.

In the present study, five AMF isolates belonging to different *Rhizophagus* species were cryopreserved for 6 months at −130 °C following encapsulation-drying and compared to the same isolates maintained on root organ cultures (ROC) at 27 °C. The morpho-anatomy (extraradical mycelium development, hyphal growth, production of branched absorbing structure (BAS), anastomosis, spore production and size, and root colonization), physiology (phosphatase activity), and genetic stability (evaluated by amplified fragment length polymorphism) are compared and discussed.

Material and methods

AMF species and isolates

Five in vitro-produced AMF isolates were considered: *Rhizophagus* sp. isolates MUCL 41835 and MUCL 43204 (Schüßler and Walker 2010), *Rhizophagus irregularis* MUCL 43194 (B1aszk, Wubet, Renker and Buscot) (Schüßler and Walker 2010), *Rhizophagus fasciculatus* MUCL 46100 ((Thaxt) Schüßler and Walker 2010), and *Rhizophagus diaphanus* MUCL 49416 (Morton and Walker) (Schüßler and Walker 2010).

ROC of the five isolates were established in association with Ri T-DNA-transformed carrot (*Daucus carota* L.) roots clone DC2 on the modified Strullu-Romand (MSR) medium (Declerck et al. 1998) solidified with 3 g l^{-1} phytagel (Sigma Aldrich, USA), following the method detailed by Declerck et al. (1998). The Petri plates were incubated in an inverted position in the dark at 27 °C. After 5 months, several hundreds to thousands of spores were obtained in each Petri plate.

Cryopreservation procedure

For each AMF isolate, propagules (i.e., a mixture of extraradical spores and root fragments containing spores/vesicles—see Lalaymia et al. 2012) were cryopreserved at −130 °C following the encapsulation-drying protocol developed by Lalaymia et al. (2012). Briefly, the propagules were isolated from 5-month-old cultures and encapsulated in alginate beads. The beads were subsequently incubated overnight in trehalose (0.5 M), dried during 48 h at 27 °C, and placed in a freezer at −130 °C, following a two-step decrease in temperature: a fast decrease (~12 °C min^{-1}) from room temperature +20 to −110 °C followed by a slow decrease in temperature (~1 °C min^{-1}) from −110 to −130 °C. Each alginate bead contained a mean of 50±5 propagules. The beads were maintained for 6 months at −130 °C. For each isolate, non-cryopreserved dried beads containing propagules were used as control and tested at the time the preserved isolates were frozen. Twenty replicates (i.e., beads) were considered per isolate and per treatment (i.e., cryopreserved or non-cryopreserved beads).

Regrowth after cryopreservation

For the non-cryopreserved treatment, the beads were placed directly after drying on 40 ml sterilized (121 °C for 15 min) MSR medium at 27 °C in the dark for propagule germination. For the cryopreserved treatment, the beads, following cryopreservation, were directly thawed in a water bath (+35 °C) and placed on the MSR medium at 27 °C in the dark as above for propagule germination.

After 4 weeks of incubation at 27 °C, to evaluate the ability of the non-cryopreserved and cryopreserved encapsulated propagules to re-initiate the fungal life cycle, beads containing germinated propagules were placed in the vicinity of transformed carrot roots, clone DC2, (two beads per root) in Petri plates (90 mm diameter) containing 40 ml MSR medium. Ten Petri plates were set up per isolate and treatment. After 5 months, spores were extracted from three randomly selected in vitro cultures of each isolate and treatment and used to re-initiate 10 mono-compartmented and 10 bi-compartmented (St-Arnaud et al. 1996) in vitro cultures with transformed carrot roots (clone DC2). After another 5 months, five mono-compartmented Petri plates were randomly selected per isolate and treatment for morphology and metabolic activity measurements. Similarly, five bi-compartmented Petri plate per isolate and treatment were used for genetic stability analysis. For each isolate,

measurements were made at 6-month intervals, first on the isolates maintained on ROC at 27 °C and then on the same isolates that had been cryopreserved for a period of 6 months at −130 °C

Morphological stability

For each isolate and treatment, the total hyphal length, the number and size of spores, the number of BAS (Bago et al. 1998), and the number of anastomosis per hyphal length (de la Providencia et al. 2005) were evaluated on the five randomly selected mono-compartmented Petri plates (i.e., replicates). The total hyphal length was assessed using a 10-mm grid marked on the bottom of each Petri plate to form 10-mm squares, following the method described by Declerck et al. (2004). Spores were counted in each square formed by the gridlines (Declerck et al. 2004). BAS and anastomosis were recorded in each Petri plate and counted following the same process as described for spores. The measurements were conducted under a dissecting microscope at magnification ×6.7 and ×40.

For each replicate, MSR medium was dissolved (Doner and Bécard 1991). Twenty spores were randomly collected from each of the five replicates and their size was measured under stereomicroscope after mounting on a microscopic slide in polyvinyl alcohol, lactic acid, and glycerin (Koske and Tessier 1983). Measurements were made with an eyepiece micrometer calibrated with a stage micrometer under a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH). Roots and extraradical mycelium were further collected from the dissolved MSR medium and percentage of root colonization was assessed following staining (Phillips and Hayman 1970). Total colonization was estimated under a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at ×50–250 magnification using the method of McGonigle et al. (1990). For each replicate, 300–350 root intersections were assessed.

Enzymatic activity

For each replicate, hyphal samples were subjected to histochemical observations for the estimation of acid phosphatase (ACP) and alkaline phosphatase (ALP) activity according to Van Aarle et al. (2002). Hyphal samples stained for ALP or ACP activity were mounted on a microscope slide with lactoglycerol. Fast blue RR granular precipitation in the hyphae was assessed using a bright-field light microscope (Olympus BH-2, Olympus Optical GmbH) at ×50 magnification. For each replicate, an approximation of 300–350 hyphal intersections was observed following the magnified intersects method (McGonigle et al. 1990). Intersections with hyphae were classified as active or non-active, depending on whether they exhibited or not fast blue RR precipitation.

Genetic stability

Amplified fragment length polymorphism (AFLP) analysis was conducted on three isolates (i.e., *Rhizophagus* sp. MUCL 41835, *Rhizophagus* sp. MUCL 43204, and *R. irregularis* MUCL 43194). Spores and hyphae in the hyphal compartment from the five replicates of each isolate established from cryopreserved and non-cryopreserved encapsulated propagules were collected by gel dissolution and pooled. The samples were frozen in liquid nitrogen in 1.5 ml tubes and ground to a fine powder using a pestle. The DNA of each sample was extracted using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was quantified by spectrophotometer (NanoDrop ND-1000, Isogen, USA). Genomic DNA was extracted twice from each non-cryopreserved isolate in order to detect variations in AFLP patterns due to differences in the DNA extraction process.

AFLP procedure

The AFLP analysis was conducted as specified by Cardenas-Flores et al. (2010) except that the restriction–ligation step for each isolate and treatment was performed on 200 ng DNA. Selective PCR was conducted with four primers couples: (1) *Eco*RI+AC-*Mse*I+T, (2) *Eco*RI+T-*Mse*I+CC, (3) *Eco*RI+AT-*Mse*I+CC, and (4) *Eco*RI+GG-*Mse*I+TA (*Eco*RI primers labeled with D4 WellRED dye, Sigma-Proligo, Beckman Coulter license, USA). To detect the resulting DNA fragments, 2 μl of the selective PCR product was run on the CEQ™ 2000 XL DNA analysis system (Beckman Coulter, USA) under the conditions cited by Cardenas-Flores et al. (2010).

The reproducibility of the resulting AFLP patterns of the non-cryopreserved isolate was confirmed by three rounds of restriction–ligation–amplification reactions for each isolate on two different DNA extractions: two independent rounds of reactions from the first and one from the second DNA extractions as described by Müller et al. (2007). A fragment position was counted as non-reproducible and excluded if it appeared in only one or two of the three replicated non-cryopreserved AFLP patterns. To estimate the technical error generated by the AFLP procedure, genomic DNA of *Rhizophagus* sp. MUCL 43204 was submitted to three independent rounds of restriction–ligation–amplification with all the primer combination described above.

AFLP data analysis

The fluorescently labeled amplified fragments were analyzed automatically with the fragment analysis software (CEQ 8000,

Beckman Coulter, USA) and corrected manually. Polymorphic DNA fragments were scored by the program as 1 for presence and 0 for absence to generate a binary matrix. Only fragments with size between 80 and 400 base pairs with relative fluorescent units above 300 were scored. Fragments present in negative controls were excluded from the analysis.

In a binary matrix, the AFLP pattern of each cryopreserved isolate was compared with AFLP patterns of the three rounds of restriction–ligation–amplification reactions of its corresponding non-cryopreserved isolate with the Dice (Bonin et al. 2007) similarity coefficient and the unweighted pair group method of arithmetic averages using the XLSTAT software version 2012.6.02 (Addinsoft, 1995–2012). Fragment position that appeared to be non-reproducible in the three non-cryopreserved AFLP patterns resulting from the three rounds of restriction–ligation–amplification reactions of each isolate was excluded from comparison. The technical error rate was then calculated in respective binary matrixes under simple match (m) (Bonin et al. 2007) similarities. Dissimilarities (i.e., the variation between two profiles) were calculated as $d=(1-m)\times 100$, where $d=0$ % corresponds to identical profiles and $d=100$ % to completely different profiles.

Statistical analysis

Statistical analysis was performed with the statistical software XLSTAT software version 2012.6.02 (Addinsoft, 1995–2012). The data were subjected to one- and two-way analysis of variance (ANOVA) and nested ANOVA. The Tukey's honest significant difference was used to identify the significant differences ($P\leq 0.05$).

Results

All five cryopreserved or non-cryopreserved AMF isolates were able to colonize roots, produce new spores, and extend hyphae within medium producing BAS and anastomosis (Table 1). Spores of each isolate were morphologically similar between both treatments. Treatments had no significant effect ($P=0.681$) on the number of spores produced. Overall, the treatment had no significant effect on the number of BAS produced ($P=0.108$). However, the number of BAS produced in cultures established from cryopreserved beads containing propagules of *R. diaphanus* MUCL 49416 was significantly higher ($P=0.002$). Overall, the cultures established from the cryopreserved encapsulated propagules had a significantly lower hyphal length ($P=0.001$). However, considering the isolates independently, this effect was only detected for the cultures established from the cryopreserved *Rhizophagus* sp. MUCL 43204 ($P=0.047$). Whatever the isolate and treatment, anastomoses were observed in all the isolates. Overall, the cultures established from the cryopreserved encapsulated propagules produced a significantly higher number of anastomosis per hyphal length (in centimeter) ($P=0.019$). Nevertheless, for each isolate considered independently, no significant difference was detected. Overall, the treatment had no significant effect on the spore size ($P=0.527$) (Table 1). Similarly, color and shape of spores of each isolate were identical between the cultures established from cryopreserved and non-cryopreserved encapsulated propagules.

Whatever the isolate and treatment, intraradical structures (i.e., arbuscules, vesicles/spores, and hyphae) were observed. Overall, the percentage of roots colonization was not affected ($P=0.823$) (Fig. 1a). For each isolate considered independently, no significant difference was observed in the percentage of roots colonization following cryopreservation.

Overall, the treatment did not affect ALP ($P=0.771$) or ACP ($P=0.809$) activity of extraradical hyphae (subpanels b and c of Fig. 1, respectively). When each isolate was considered independently, no significant difference was found in the AMF metabolic activity (i.e., ALP and ACP staining) between cultures established from cryopreserved and non-cryopreserved encapsulated propagules

AFLP patterns contained between 156 (*Rhizophagus* sp. MUCL 41835) and 206 (*Rhizophagus* sp. MUCL 43204) DNA fragments. Isolates displayed a different AFLP pattern between cryopreserved and non-cryopreserved treatments but within the range of the technical error (i.e., 4.4 %). After cryopreservation, among the 206 fragments obtained with the four couples of primers, the *Rhizophagus* sp. MUCL 43204 pattern was similar at 98.8 % to the non-cryopreserved pattern. The *Rhizophagus* sp. MUCL 41835 isolate presented a similarity of 98.5 % between cryopreserved and non-cryopreserved patterns. For *R. irregularis* MUCL 43194, a similarity of 95.8 % was obtained between the patterns of the two treatments (Supplementary Fig. 2).

Discussion

Here we demonstrated that, with few exceptions, all the cryopreserved *Rhizophagus* isolates exhibited the same morpho-anatomy as the non-cryopreserved isolates. As previously reported by Lalaymia et al. (2012) for encapsulation-drying, subsequent cryopreservation for 6 months at −130 °C of the five in vitro-produced AMF isolates did not impact their survival (i.e., germination within beads), root colonization, and spore production. Similarly, the daughter spores isolated from these cultures were able to re-associate a carrot root in vitro and to reproduce the fungal life cycle either in mono- and bi-compartmented Petri plates. Extraradical mycelium developed as compared to the cultures maintained at 27 °C. The five AMF isolates were able to colonize the roots and produce

Table 1 Number of spores, number of branched absorbing structure, hyphal length, number of anastomosis per hyphal length, and spore diameter of five arbuscular mycorrhizal fungi grown in root organ culture following propagule encapsulation in alginate beads and drying cryopreservation at −130 °C as compared to non-cryopreserved encapsulated-dried isolates

Isolate	No. of spores		No. of branched absorbing structures		Hyphal length (cm)		No. of anastomosis per hyphal length (cm)		Spore diameter (μm)	
	Non-cryopreserved (control)	Cryopreserved	Non-cryopreserved (control)	Cryopreserved	Non-cryopreserved (control)	Cryopreserved	Non-cryopreserved (control)	Cryopreserved	Non-cryopreserved (control)	Cryopreserved
Rhizophagus sp. MUCL 41835	9,552±3,620a	9,861±3,546a	4,165±672a	4,481±973a	4,668±1,450a	3,215±698a	0.17±0.07a	0.19±0.05a	(30)–71–(116)a	(40)–72–(118)a
Rhizophagus sp. MUCL 43204	10,408±1,150a	10,685±2,061a	4,270±819a	3,477±517a	4,099±1,001a	2,828±681b	0.19±0.06a	0.21±0.06a	(32)–69–(116)a	(40)–72–(118)a
Rhizophagus irregularis MUCL 43194	5,931±1,395a	6,951±2,811a	2,807±469a	3,118±574a	2,751±665a	2,590±1,095a	0.12±0.02a	0.18±0.09a	(42)–81–(124)a	(40)–76–(116)a
Rhizophagus fasciculatus MUCL 46100	6,131±1,890a	4,053±1,555a	2,150±526a	2,044±607a	3,355±870a	2,751±650a	0.27±0.06a	0.33±0.09a	(20)–41–(80)a	(18)–42–(76)a
Rhizophagus diaphanus MUCL 49416	4,647±2,026a	3,777±1,008a	1,133±308a	2,945±864b	3,684±377a	3,130±449a	0.34±0.08a	0.44±0.07a	(28)–49–(66)a	(32)–46–(74)a

For each parameter, values in the same line followed by identical letter did not differ significantly ($P \leq 0.05$, Tukey's HSD). Data represent the means of five replicates (mean ± SE). For spore diameter, values in the same line followed by identical letters did not differ significantly (nested ANOVA $P \leq 0.05$, Tukey's HSD). Data represent means and size distribution of diameter measurements based on 100 spores from five different replicates (i.e., 20 spores per replicate)

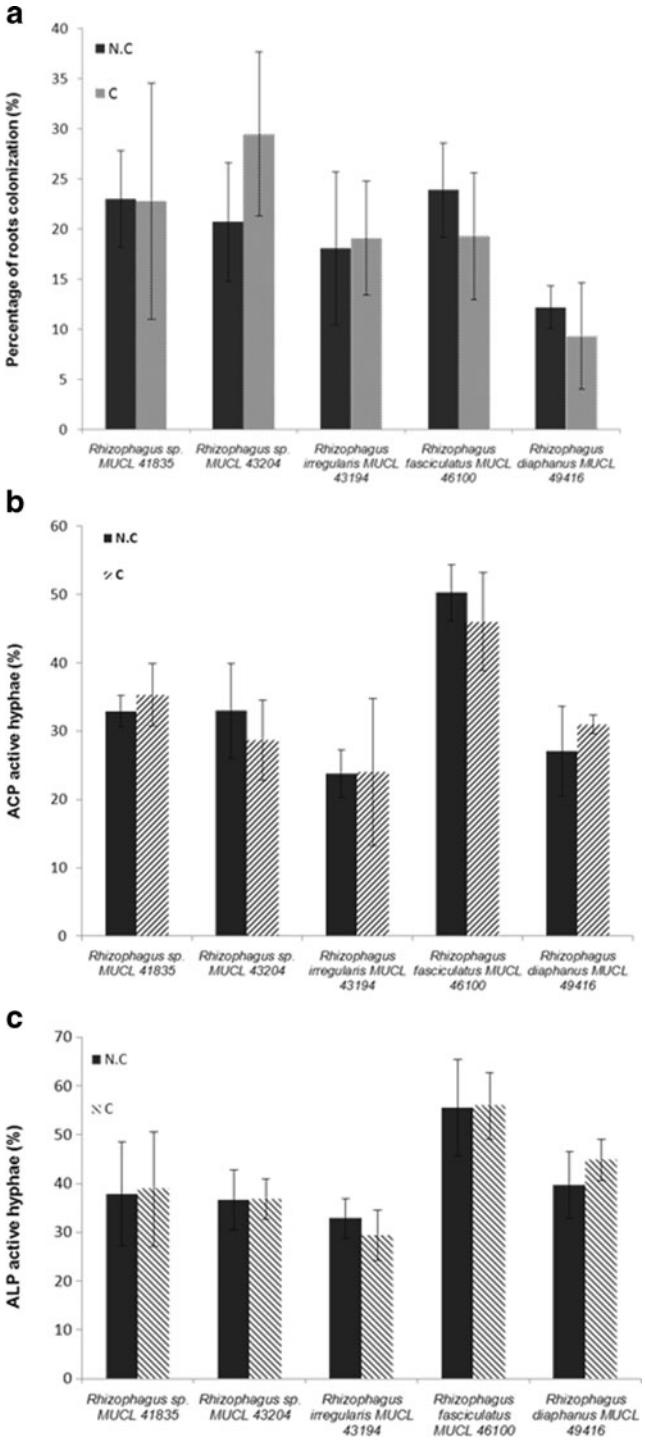

Fig. 1 Percentage of carrot root colonization (**a**) proportion of alkaline phosphatase activity (**b**) and acid phosphatase activity (**c**) in the extraradical hyphae of five AMF isolates cryopreserved (C) for 6 months at −130 °C or maintained on root organ culture (i.e., non-cryopreserved (NC) treatments) at 27 °C. Mean value (± standard error, $n=5$) followed by the same letter, within a column, did not differ significantly ($P \leq 0.05$; Tukey's HSD)

typical intraradical structures (i.e., arbuscules, vesicles/spores, and intraradical hyphae). Similarly, an extensive extraradical mycelium was produced supporting spores, BAS, and anastomosis. With the exception of *Rhizophagus* sp. MUCL 43204, the hyphal lengths in cultures established from cryopreserved encapsulated propagules were identical to those obtained from non-cryopreserved encapsulated propagules and were consistent with previous data reported on *Rhizophagus* species (de Jaeger et al. 2011) but were larger as compared to the values reported by de la Providencia et al. (2005) with the same isolate. The reason for such a difference may be attributed to the intrinsic heterogeneous growth of AMF in vitro. Indeed, in several studies, it has been reported that growth dynamics may greatly vary from replicate to replicate within a same isolate (Declerck et al. 1996, 2001, 2004). Nevertheless, this difference did not affect the fungal growth and the development of a profuse hyphal network supporting BAS, spores, and anastomosis. Whatever the isolate and treatment, spores were produced on hyphae of primary, secondary, and lower order as well as associated to BAS, and no significant difference was found in the number of spores produced between treatments. Identical values were reported by de la Providencia et al. (2005) and Lalaymia et al. (2012) for the same isolates maintained by regular subcultivation and following cryopreservation, respectively. BAS structures, reported as the preferential sites for mineral nutrient acquisition (Bago et al. 1998, 2004), were also produced in large quantities. BAS number was only significantly higher in cultures established from cryopreserved encapsulated propagules of the *R. diaphanus* MUCL 49416 isolate as compared with the control. As above, the difference may be related to the variability in BAS number between replicates of a same isolate (Declerck et al. 1996, 2001, 2004) or to the difficulty to distinguish BAS within the profusely branched fungal colony. Anastomosis, that is the process of fusion between branches of the same or different hyphae to produce a mycelial network (Kirk et al. 2001), was also observed in the extraradical hyphae for all the isolates and treatments under study. Whatever the isolate and treatment, the number of anastomosis per hyphal length recorded here was much lower than the number observed by de la Providencia et al. (2005) and Voets et al. (2006). This difference could be influenced by cultivation systems due to the variation in physiological processes and/or gene expression (Purin and Morton 2011). Whatever the isolate and treatment, ALP and ACP activity was observed in the extraradical hyphae as reported earlier in other systems (Joner and Johansen 2000; Van Aarle et al. 2001, 2002; Ezawa et al. 2001; Olsson et al. 2002; Aono et al. 2004; Zocco et al. 2011). Cryopreservation did not affect phosphatase activity.

Finally, when genetic stability of the AMF cultures was assessed using AFLP fingerprinting (Vos et al. 1995), a technique used to assess the stability of animal cells, plant, and fungi after maintenance by cryopreservation (Hsu et al. 2008; Mikula et al. 2011; Voyron et al. 2009), no differences in AFLP patterns were observed within three isolates

maintained for 6 months by cryopreservation or maintained on ROC at 27 °C, outside the range of technical error. This suggested, that in the time frame of conservation, the genomic stability of the isolates was maintained. Using AFLP, Cardenas-Flores et al. (2010) also did not observe any genetic variation in different clonal lineages of *R. irregularis* MUCL 43194 originating from the same ancestor culture and maintained by serial sub-cultivation for at least 70 generations under different growth conditions.

In conclusion, the present results demonstrate for the first time that in vitro-produced AMF isolates that are cryopreserved at −130 °C for 6 months maintain their morphology, physiology, and genetic stability, as compared to the same isolates maintained on ROC at 27 °C. Consequently, the cryopreservation protocol developed here for AMF isolates belonging to the genus *Rhizophagus* (i.e., *R. irregularis*, *R. fasciculatus*, *R. diaphanous*, and two undefined isolates) appears to be a suitable method for maintaining these obligate symbionts, decreasing the necessity for laborious subcultivations. These results are of particular importance for culture collections, where cryopreservation can represent a preferred means to preserve the fungal organisms. The protocol of cryopreservation and subsequent effects on fungal characteristics needs to be extended/adapted to other species and genera and for longer periods of time to validate this technique over time and for a large set of AMF.

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